DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Molecular characterization of phytoprotector

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Ana Catarina Gomes (Unidade de Genómica - Biocant) e do Professor Doutor Jorge Canhoto (Universidade de Coimbra)

Valéria dos Santos Custódio

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Este trabalho foi desenvolvido e financiado pelo projeto "InovWine II", uma iniciativa QREN, do financiamento EU/FEDER, através do COMPETE – Programa Operacional Fatores de Competitividade, com a referência FCOMP-01-0124-FEDER-027411.

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Ao meu pai, Osvaldo, à mama “prossora” e ao meu tio Valério

Agradecimentos

A realização dessa tese não seria possível, se não fosse o apoio de pessoas importantes e essenciais.

Ao meu pai, Osvaldo e à Mama "prossora" um muito obrigado por estarem sempre presentes apesar da distância, e tudo o que alcancei até hoje foi por querer ser igual a vocês. E ao meu tio, Valério pelo apoio e carinho durante toda a minha vida académica.

À Ana Catarina Gomes, por todo o apoio, e por tornar possível não só a realização da tese como do Mestrado. O meu melhor desempenho no desenvolvimento deste trabalho e a minha gratidão será sempre uma certeza.

Ao professor Jorge Canhoto, por me ter aceite ser meu orientador e pelo apoio e disponibilidadade. E principalmente por ter contribuído nessa importante fase de crescimento.

À Cátia Pinto, pelo apoio, dedicação e principalmente pela amizade. Aprendi contigo o que de melhor se pode aprender com a ética de trabalho. Ensinaste-me o verdadeiro significado de paciência, cuidado, esforço, empenho e audácia no trabalho.

As minhas "tweens" Claudinete e Claudete Neves, um obrigada é tudo que consigo dizer, mais vem acompanhada de mais sentimentos e palavras que talvez precisam de ser inventadas, para demonstrar o meu agradecimento por tentarem manter a minha sanidade mental durante esses 14 anos de convívio.

À Cátia Santa, nunca poderei esquecer-me de ti por todo o apoio nos momentos que mais precisei. À Joana Fernandes pela amizade e pelas conversas, a tua amizade será sempre especial. Aos meus colegas de laboratório, principalmente à Raquel Santos e ao Daniel Duarte pela amizade, apoio e dedicação

E como não podia deixar de ser, a todos do Biocant, principalmente à Dona Alda, por as vezes conseguirem que eu me sentisse em casa.

A todos aqui, um muito obrigado.

Resumo

A videira (*Vitis vinifera*) é uma das espécies mais susceptível a fitopatogéneos (bactérias, fungos, nematóides e vírus). Normalmente, no combate desses fitopatogéneos são utilizados compostos químicos. No entanto, têm-se verificado nos últimos anos um crescente interesse da utilização de agentes de controlo biológico como método alternativo para a redução ou substituição desses compostos químicos.

Neste sentido, o objetivo deste trabalho consiste no isolamento, seleção, caracterização e identificação de microrganismos antagonistas endógenos isolados de vinhas localizadas na região da Bairrada. Foram obtidos um total de 354 microrganismos, isolados de amostras de solo, raiz, caule, folhas, uvas e mosto e as técnicas moleculares permitiram identificar 140 fungos filamentosos. Os restantes 214 isolados caracterizados como bactérias e leveduras foram sujeitas ao teste de antagonismo contra 8 fungos fitopatogéneos selecionados. E um total de 72 isolados (34%) apresentou atividade antagonista significativo.

Estes isolados foram testados quanto à sua capacidade de produção de sideróforos e solubilização de fosfato. Dos 72 microrganismos testados, um total de 31 isolados apresentaram simultaneamente reações positivas para a solubilização de fosfato e produção de sideróforos. E os isolados mais promissores como agentes de controlo biológico apresentaram ser sensíveis aos agroquímicos comerciais. Três espécies destacaram-se como potenciais promotores de crescimento vegetal e promissores agentes de controlo biológico, pois não só apresentaram uma inibição micelial significativa como foram capazes de solubilizar fosfato e produzir sideróforos simultaneamente.

De um modo geral, as características moleculares, bioquímicas e metabólicas dessas espécies torna-as potenciais promotores de crescimento vegetal e promissores agentes de controlo biológico na vinha Além de contribuírem para a redução dos agroquímicos, e permitirem o desenvolvimento de uma viticultura sustentável e orgânica.

Palavras-chaves: *Vitis vinifera*, Fitoprotetores, Biocontrolo, antagonismo, Agroquímicos

Abstract

Grapevine is susceptible to a diverse phytopathogens attack that compromises its cultivation and is responsible for considerable losses of yield and therefore will affect wine quality. The control of these phytopathogenic microorganisms is based on the use of phytochemical products that are hazardous to environment and are responsible for the emergence of resistant strains. In order to reduce the doses of phytochemicals applied in viticulture, a recent interest has been focus in new ecological alternatives also known Biocontrol.

In this context, the aim of this work was to isolate, identify and characterize endogenous microorganisms isolated from vineyards located in Bairrada, Portugal. A total of 354 microorganisms were isolated from soil, roots, leaves, stems, grape, and must samples and the molecular tools allowed to identify 140 filamentous fungi. The remaining 214 bacterial and yeast isolated were tested for *in vitro* antagonism assays against 8 pathogenic fungi. And a total of 72 isolates (34%) exhibited a significant antagonistic activity.

The antagonistic isolates were also evaluated for their ability for siderophore producing and capacity to solubilize phosphate. Among these, 31 isolates were able to simultaneously produce siderophores and solubilise phosphate. And the most promising isolates were generally showed very sensitive towards trade formulates agrochemicals. Furthermore, three isolates highlighting as potential biological agents and plant-growth promoters by showed a significant inhibition mycelial (≥50%), and exhibited simultaneously a positive reaction for both production of siderophores and solubilize phosphate.

Overall, due to the molecular, biochemical and metabolic characteristics of these species make them as potential protectors of grapevine against fungal diseases. Furthermore, they can also contribute to a significant reduce of agrochemicals and favor the production of a sustainable agriculture.

Keywords: *Vitis vinifera*, Phytoprotector, Biocontrol, Antagonism, Agrochemicals

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List of abbreviations

BCA: Biological Control Agents

BLASTn: Basic Local Aligment Search Tool Nucleotide

CAS: Chrome Azurol S

CTAB: Cetyl Trimethylammonium Bromide

DNA: Desoxyrribonucleic Acid

dNTPs: Deoxynucleotide triphosphates

EDTA: Ethylene diamine tetra acetic acid

HA: hectares

ITS: Internal Transcribed Spacer

M: Molar

mM: Milimolar

µM: Micromolar

OIV: International Organisation of Vine and Wine

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar

PGPB: Plant Growth Promoting Bacteria

rDNA: ribosomal DNA

rpm: Revolutions Per Minute

TAE: Tris – Acetate - EDTA

TE: Tris - EDTA

YEPD: Yeast Extract Peptone Dextrose

1 - Introduction

1. Inside the vineyard – phytoprotectors and their importance for a sustainable viticulture.

1.1. Viticulture in Portugal

Viticulture is one of the key economic activities in Europe. In the last report of the International Organisation of Vine and Wine (OIV), the production of wine in Europe was estimated at 62,3% across 4 mHA cultivated, which represents the largest area of vineyards in the world (OIV, 2013).

Of all European countries, Portugal has the largest percentage of area under vines. Indeed, viticulture is one of the most dynamic sectors of Portuguese agriculture and the species *Vitis vinifera* is the most cultivated due to its high quality for the production of wine (Ferreira *et al.*, 2004). However, this species is highly susceptible to an array of pathogens as fungi, bacteria, viruses and nematodes which may cause serious problems to the vitality of plantations and consequently jeopardize the economy of wine sector. Among these microorganisms, fungal pathogens are the most damaging in vines cultivation. In fact, fungi pathogens cause significant losses in the wine industry because they could infect the plant, thus reducing its vitality and productivity, or could directly infect the berries. Consequently, the yield and wine quality is compromised (Fraga *et al.*, 2012).

The most threatening fungal diseases of grapevine are downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*) and gray mold (*Botrytis cinerea*) which are mainly controlled by the application of chemical fungicides to reduce the incidence of diseases (Ferreira *et al.*, 2004; Barata *et al.*, 2012).

According to the Direcção Geral da Agricultura e do Desenvolvimento Rural (DGADR), in 2010, more than 68 % of the agrochemicals marketed in Portugal were fungicides from different chemical families (DGADR, 2011).

In the past few years, these fungicides have been successfully used for the control of pests and diseases in grapevine. However, they have been losing their effectiveness as some pathogens strains have developed genetic resistance to these compounds (Compant *et al.*, 2005a). In addition, synthetic fungicides cause an undesirable effect on the microbial diversity of agroecossystems affecting both phytopathogenic and beneficial microorganisms (Pinto *et al.*, 2014). Furthermore, also the soil fertility is negatively affected, which influences the growth of plants (Cañamás *et al.*, 2011; Furuya *et al.*, 2011).

Due to the increase of awareness to sustainable practices, there has been an increasing interest of exploring new alternative methods for controlling diseases through the application of environmental friendly strategies. Thus, biological control has emerged as an environmental friendly alternative to chemical pesticides, to reduce the doses of chemicals applied and pathogens strains resistant in viticulture (Compant *et al.*, 2005a).

1.2. Biological control

The biological control, also denominated as Biocontrol, can be defined as the use of non-pathogenic microorganism to reduce the incidence of infections caused by pathogenic microorganisms, to stimulate plant growth and to reduce biotic and abiotic stresses of plant (Ait Barka *et al.*, 2002; Compant *et al.*, 2010). These non-pathogenic microorganisms are commonly designated phytoprotectors or biological control agents (BCA).

The biological control is characterized by different types of interactions between plant and BCAs that colonize soil, roots, tuber, stems, leaves and other plant organs. The mutualism, commensalism, neutralism, competition, amensalism, parasitism, predation and antagonism are some of the interactions that occur between plants, phytopathogen and phytoprotector microorganisms (Berg, 2009; Heydari and Pessarakli, 2010).

Regarding the control of plant's pathogenic microorganisms, the antagonism is the dominant interaction. According to Heydari and Pessarakli (2010), antagonism is defined by the interaction of two or more species where one microorganism is harmed in relation to another, or even both are harmed. This interaction between species can result in a reduced growth, activity and fertility of the interacting microorganisms. Various authors have considered the mechanism of antagonism as the most common of biological control because the BCAs microorganisms compete for nutrients and space with the plant's phytopathogens (Dardanelli *et al.*, 2010; Figueiredo *et al.*, 2010).

1.3. Mechanisms involved in biological control

The biological control can be achieved by multiple mechanism of action (Figure 1) which could be direct or indirect. The direct mechanisms are based on the production of substances which promote plant growth and increase nutrient availability in soil for plants by transforming these nutrients in to a simpler form of assimilation. The indirect mechanisms consist in the suppression of plant pathogens (Akhatar and Siddiqui, 2010; Ribeiro and Cardoso, 2012).

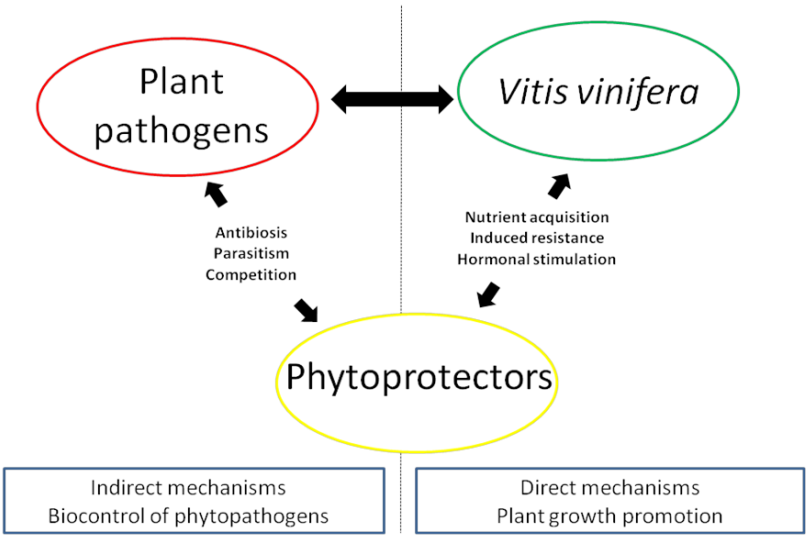

Figure 1. *Vitis vinifera* – microbe interactions.
Mechanisms involved in biocontrol of plant pathogens (adapted from Berg, 2009).

The direct promotion of plant growth requires the provision of some compounds synthesized by BCAs microorganisms through the production of siderophores and the

hormone indole-3-acetic acid (IAA). Its impact on plant growth is due to the greater absorption of nutrients from the surrounding environment, in particular to the capacity of phosphate solubilisation or nitrogen fixation by BCAs microorganisms (Bloemberg and Lugtenberg, 2001; Whipps, 2001; Lodewyckx *et al.*, 2002; Compant *et al.*, 2005; Compant *et al.*, 2005a; Ahmad *et al.*, 2006; Adesemoye *et al.*, 2009; Compant *et al.*, 2010; Heydari and Pessarakli, 2010).

The indirect promotion occurs when phytoprotectors inhibit the growth of plant pathogenic microorganisms through the diffusion of antibiotics, volatile organic compounds, toxins and biosurfactants for surrounding areas. This promotes a process designed as antibiosis (Berg, 2009). Besides these, the competition for space and nutrients (Compant *et al.*, 2005a; Correa and Soria, 2010), production of extracellular enzymes such as chitinase and β - 1,3 – glucanase, which degrade the cell wall of fungi (Heydari and Pessarakli, 2010), and the induction of systemic resistance (SR) in plants, which increases the defensive response, are all processes that contribute to the inhibition of phytopathogenic attacks (Verhagen *et al.*, 2010).

1.3.1. Siderophores production

Iron is an essential element which plays an important role in many biological processes such as photosynthesis, nitrogen fixation, methanogenesis, hydrogen production and consumption, cellular respiration, oxygen transport, gene regulation and DNA biosynthesis (Yuan *et al.*, 2001; Andrews *et al.*, 2003).

Despite being the fourth most abundant element in the earth's crust, iron bioavailability is extremely limited in aerobic environments (in the presence of oxygen and neutral pH), because ferric iron (Fe^{3+}) reacts with oxygen to form insoluble ferric hydroxides (FeOOH) (Loper and Buyer, 1991). In a soil with an iron-limited condition, microorganisms produce siderophores to solubilise environmental iron, capturing and transporting extracellular inorganic iron to the cell or, in the case of being produced intracellularly, to iron storage (Johnson, 2008).

Siderophores are low molecular weight compounds (< 1000 Da), highly electronegative and with high affinity towards Fe^{3+} (Andrews *et al.*, 2003). They usually

form hexadentate octahedral complexes with ferric iron and typically employ hydroxamates, α-hydroxycarboxylates and catechols as extremely effective Fe^{3+} ligands (Alexander and Zuberer, 1991).

It has been suggested that the excretion of siderophores forming ferric ion complex (Fe^{3+} -Siderophore complex), which is transported into the cell via specific channels, allows the metal availability and stimulates the plant growth by improving the Fe nutrition of the plant, and by depriving pathogenic fungi (Alexander and Zuberer, 1991; Compant *et al.*, 2005a; Akhatar and Siddiqui, 2010).

1.3.2. Phosphate solubilisation

Plants need several macro and micro nutrients for their growth and reproduction. Phosphorus (P) is a macronutrient essential for plant growth and biological development (Kaymak, 2010). Most agricultural soils contain high concentrations of phosphorus as a result of the application of phosphate fertilizers, which leads to the accumulation of this element. However, a large portion of soluble inorganic phosphate applied to soil, presented in chemical fertilizers, is rapidly immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} thus becoming unavailable to plants (Pradhan and Sukla, 2006). The phenomena of fixation of phosphate in soil are highly dependent on pH and soil type.

Microbial communities have been recognized as strong candidates for solubilisation and subsequent absorption of phosphate, promoting the uptake of this element for plants (Yang *et al.*, 2009). These beneficial microorganisms are involved in a framework of interactions in the soil and they convert insoluble phosphate into a soluble form by acidification, chelation and exchange reactions in the periplasm (Pradhan and Sukla, 2006; Oliveira *et al.*, 2009; Prasanna *et al.*, 2011). Microrganisms belonging to the genera *Acinetobacter, Archrobacter, Bacillus, Enterobacter, Erwinia, Klebsiella, Mesorhizobium, Microccus, Pseudomonas* and *Rhizobium* have been reported as efficient phosphate solubilisers (Rodriguez and Fraga, 1999; Sridevi *et al.*, 2007; Desai *et al.*, 2012).

1.4. Importance of the identification and characterization of microorganisms involved in the biological control

The identification and characterization of phytoprotector microorganisms involves a phenotypic characterization obtained by morphological, physiological or biochemical analysis and genotypic identification through molecular methods.

1.4.1. Phenotypic characterization

Phenotypic characterization of microorganisms involves the analysis of their morphological, physiological or biochemical properties (Pinto, 2011). Traditionally, the morphological characterization comprises a colony morphology analysis as color, dimensions and form of microorganisms and microscopic appearance of the cells (shape, endospore, flagella, and inclusion bodies). While these tests consider the morphological and physiological properties of microorganisms, the biochemical characterization is related to the ability of microorganisms to metabolise different substrates in different concentrations of salinity, to grow under different pH and temperature conditions and their susceptibility toward different kinds of antimicrobial agents. Other factors such as concentration of oxygen, fermentation reactions and metabolism of nitrogen are also considered.

Although these tests allow for the detailed characterization of strains, some drawbacks have arised from the fact that microorganisms of the same species have different phenotypic characteristics in different environmental conditions (Figueiredo *et al.*, 2010). Thus, it becomes very important to complement these studies with other methods such as molecular characterization.

1.4.2. Molecular characterization

The study of the interactions between plants and microorganisms (phytopathogenic and phytoprotectors communities) are important for a better understanding of many ecological processes and functioning of ecosystems (Singh *et al.*, 2004).

In the study of bacterial communities, the sequencing of 16S rDNA showed to be useful for species identification as well as determination of phylogenetic relationships. As 16S rDNA is a highly conserved region it has become an important tool both for species identification and to study bacterial phylogeny and evolution (Janda and Abbott, 2007).

For the identification and analysis of the phylogenetic relationships of eukaryotes, all genes encoding ribosomal subunits (18S, 5.8S and 26S) and non-coding regions (ITS1 and ITS2) are used. The Internal Transcribed Spacer region (ITS) is the most applied for the identification of fungi and yeasts microorganisms. This region consists of the non-coding regions ITS1 (rapid evolution) and ITS2 (suffers fewer mutations than ITS1) separated by the 5.8S gene (conserved region) (Nilsson *et al.*, 2008). This region is located between the 18S gene (small subunit- SSU) and 28S gene (Large Subunit- LSU) (Bellemain *et al.*, 2010). The size of ITS region varies from organism to organism and typically ranges between 450 and 700 bp. The large number of ITS copies per cell and is highly conserved and variable regions, makes these region an appealing target for sequencing environmental substrates.

2 - Objectives

The application of agrochemicals, such as fertilizers and pesticides, in viticulture can be considered one of the factors that influence the dynamics of microbial community in vineyards and contribute to the imbalance of these communities (Schmid *et al.*, 2011; Martins *et al.*, 2012). Thus, it becomes important to uncover the structure of microbial communities that naturally colonize the grapevine to better understand the interactions that occur between microorganisms (both patogenic, neutral and beneficial) with the host plant.

Unveiling this microbial communities and its interactions will be a step forward to better understand some microbial mechanisms such those involved in plant infection, plant growth promotion or pathogen defense through biological control (Compant *et al.*, 2005a; Berg, 2009).

In this context, the major goals of this work were to screen, identify and characterize endogenous microorganisms isolated from grapevines from Bairrada region. Regarding this, the specific goals were:

- To Isolate and molecular identify microbial microorganisms, both phytoprotectors and plant pathogenic fungi, isolated from different vineyards from Bairrada region, during the vine campaigns of 2011 and 2012;
- To carry out *in vitro* analyses of the microbial interaction between phytopathogens and potential antagonist microorganisms;
- To evaluate the ability of the selected isolates to solubilize phosphate and to produce siderophores.
- To evaluate the tolerance of antagonistic microorganisms to agrochemicals used for vineyard's treatment through an *in vitro* screening with culture media supplied with different concentrations of the active element of each agrochemical.

3 – Materials and Methods

3.1. Sampling site

The collection of biological samples was carried out in four different vineyards, located in the Bairrada region and selected by the Genomics Unit from Biocant. One of these vineyards was abandoned and vines were untreated. For each vineyard, the sampling points was randomly assigned and samples such as soil, roots, leaves, stems and grapes of both healthy and diseased grapevine have been collected from Arinto, Baga, Bical, Maria Gomes, Tinta Roriz and Touriga Nacional varieties. These samples were collected during the vine campaign of 2011 (April to September) and September 2012. Also must samples were analysed. These samples were collected from different stages of wine fermentation performed in a laboratory-scale fermentation. For all samples collected microorganisms were immediately isolated, through classic microbiology techniques currently used in the laboratory.

3.2. Isolation of microorganisms

As referred above, the microorganisms were isolated from different parts of grapevine as roots, leaves, stems and grapes. And the soil also has been collected from the isolation. For the isolation of the microorganisms present in the leaves two different strategies were carried out. The first consisted of placing portions of the leaves (healthy and displaying disease symptoms) directly on the two culture medium used - Potato dextrose agar (PDA) and Yeast extract peptone dextrose (YEPD) (Annex I). In the second, leaves were cut in small sections (10 mm) followed by surface sterilization during 20s in a 2% (w/v) sodium hypochloride solution and washed with sterile water. The segments were then macerated in a sterile NaCl (0.85%) solution. Serial dilutions were performed and microorganisms were cultivated on the same culture medium as referred above. The second strategy was also applied to roots and stems. For soil

samples, 0.1 g of soil from each sample was weighed, suspended in a sterile NaCl (0.85%) solution and shaken. Then the soil suspension was serially diluted by pipetting 1 ml aliquots into 9 ml of NaCl (0.85%) solution and cultured in Petri dishes on potato dextrose agar (PDA) medium and yeast extract peptone dextrose (YEPD) (Annex I). Bacteria and yeast were grown at 30ºC for 48/72h and the filamentous fungi at 28ºC, for 15 days. For each strain, a specific code was assigned according to the nomenclature adopted by the laboratory and cryopreserved at -80ºC in duplicate. Bacteria and yeasts were preserved in 40% and 80% of glycerol, respective with the corresponded broth growth medium. Filamentous fungi were cryopreserved in 20% of glycerol supplemented with potato dextrose broth (PDB) (Annex I).

3.3. Molecular identification

3.3.1. DNA extraction of bacteria and yeasts

The DNA extraction of bacterial and yeasts isolates, and the consequently the molecular identification was carried out only for those isolates that showed the major biocontrol activity against the selected phytopathogenic fungi. The kit Promega Genomic DNA (Promega, USA) was used for DNA extraction. Isolates with approximately 48h of growth in PDA plates were used, and cells were removed from the plate, and added to 480 µl of EDTA (50 mM) (Annex II) and 120 µl of lysozyme (10 mg / ml), and incubated at 37°C for 30-60 minutes. Samples were then centrifuged for 2 minutes at 13000 - 16000g, the supernatant was discarded and 600 µl of Nuclei Lysis solution was added to the pellet and incubated at 80°C for 5 minutes. After this, samples were allowed to cool at room temperature and 3 µl of RNase solution was added. The solution was homogenized by inverting the eppendorfs 2-5 times and incubated at 37°C during 60 min. Samples were kept at room temperature and after cooling 200 µl of Protein Precipitation Solution was added and each eppendorf was vigorously vortexed for 20s. Subsequently, the eppendorfs were placed on ice for 5 minutes and centrifuged at 13000 - 16000g for 3 minutes. The supernatant was transferred to a new 1.5 ml eppendorf which contained already 600 µl of isopropanol at room temperature. This was mixed thoroughly by inversion and allowed to precipitate the DNA. The samples were centrifuged at 13000 -

16000g for 2 minutes and the supernatant was discarded. Then a wash was carried out with 600 µl of 70% ethanol and inverted gently several times and centrifuged at 13000 - 16000g for 2 minutes. The supernatant was discarded and the pellet was dried in the speed vacuum (DNA 120 Speedvac concentrator, USA) or in the laminar flow chamber (Mars safety Class 2) for 10-15 min. The DNA pellet was ressuspended in 50-100 µL of DNA rehydration solution and incubated overnight at 4°C. The DNA was then quantified in NanoDrop (Nanodrop ND - 100) and stored at -20°C until further use.

3.3.2. DNA extraction of filamentous fungus

Cultures of filamentous fungus (7-15 days of culture) were used for DNA extraction. Eppendorfs were previously prepared with approximately 200 µl of rehydrated glass beads and a portion of fungi mycelium was sliced for DNA extraction. Then 400 µl of preheated 2x CTAB buffer at 65ºC was added and the eppendorfs were vortexed. To allow the mechanical breaking of fungi cells, 2 cycles of 5 minutes at maximum frequency (30 Hz) in tissue lyser was applied. Between cycles, samples were allowed to rest on ice for 2 minutes. Samples were centrifuged at 10000 rpm for 10 minutes at 15°C and the supernatant was collected to a new eppendorf. The mechanical breaking of cells was repeated twice with 300 µl of 2x CTAB buffer and a cycle of 60 seconds in tissue lyser. These samples were also centrifuged at 10000 rpm for 10 min and the supernatant was added to the above eppendorf. The samples were incubated at 65°C for one hour and then centrifuged at 13000 rpm for 5 min. The supernatant (300 µl) was transferred to another eppendorf and 600 µl of chloroform were added. The mixture was homogenized by inversion. After centrifugation at 13000 rpm for 5 min, the supernatant was carefully collected to a new eppendorf and 750 µl of cold isopropanol (-20ºC) were added. The mixture was gently homogenized by inversion. Then the eppendorfs were placed at -20°C for at least 2h or overnight to allow the precipitation of DNA. Samples were centrifuged at 13000 rpm for 30 minutes at 4°C and the supernatant was discarded. Subsequently, 200 µl of 70% ethanol (-20°C) were added and stirred for 2 minutes. The eppendorfs were centrifuged at 7000 rpm for 5 minutes, the supernatant was removed and the DNA pellet was dried by the speed vacuum (DNA 120 Speedvac concentrator, USA) or in the laminar flow chamber for 10-15 min. The DNA was the ressuspended in 50-100 µl of 1X TE

buffer, quantified in the NanoDrop (NanoDrop ND - 100) and stored at -20°C until further use.

3.3.3. Amplification, purification and sequencing of the ITS and 16S rDNA regions

The internal transcribed spacer (ITS) region was used to identify all eukaryotic isolates and the amplification of the gene 16S rDNA, which encodes the 16S subunit of the ribosome (Figure 2), to identify the prokaryotic isolates. The amplification of the 16S rDNA for prokaryotic identification was used because this gene is present in almost all prokaryotes, their functions have not changed over time and is large enough for bioinformatic analysis (Janda and Abbott, 2007).

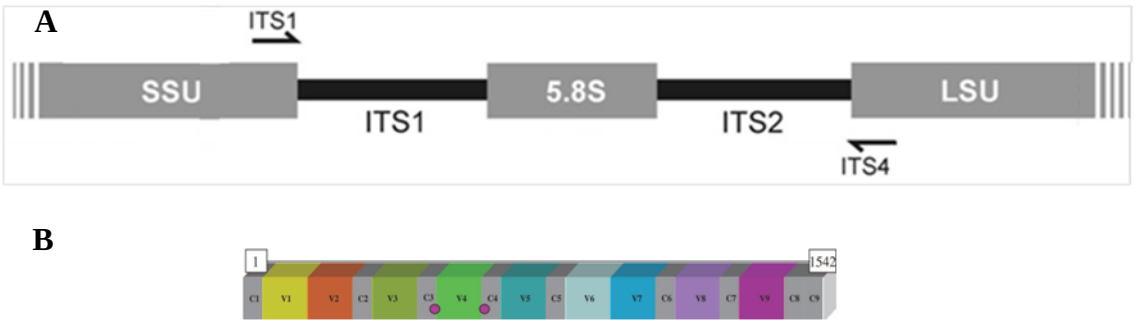

Figure 2: Regions of the DNA which were used to identify eukaryote and prokaryote.
A: ITS region and commonly primers used to amplifyind the non-coding ITS1 and ITS2 regions. B: 16S rDNA regions.

The polymerase chain reaction (PCR) reaction for ITS amplification contained: 1x Biocant buffer (Biocant, Portugal); 2 mM dNTPs (Bioron, Germany); 2 mM $MgCl_2$; 0.4 mM of each primer - ITS1 and ITS4 (**Erro! A origem da referência não foi ncontrada.**); 2.5U of Taq DNA Polymerase (Biocant); 1 or 2 µl of DNA and sterile milli-Q water for a final reaction volume of 25 µl.

The same reaction volume was used to amplify the 16S rDNA and the mixture contained: 1x Fidelity Taq buffer (Affymetrix, USB); 1.7 mM $MgCl_2$; 2 mM dNTPs; 0.8 mM of each primer - 16S_R2 and 16S_F2 (**Erro! A origem da referência não foi ncontrada.**); 1.5 U Taq Fidelity (Affymetrix, USB); 2 µl of DNA and sterile milli-Q water.

Table I: Primers used for the amplication of the ITS region and 16S rDNA.

Primers	Region	Sequence
ITS 1	ITS	5'- TCCGTAGGTGAACCTGCGG-3'
ITS 4		5'-TCCTCCGCTTATTGATATGC-3'
16S_F2	16S rDNA	5'AGAGTTTGATCCTGGCTCAG-3'
16S_R2		5'-GGYTACCTTGTTAACGACTT-3'

The PCR conditions for ITS and 16S rDNA amplification are described in detail in the **Erro! A origem da referência não foi encontrada.**. All reactions were carried out n an Eppendorf thermocycler AG (Eppendorf, USA).

Table II: PCR amplification conditions.

Region	Amplification conditions	
ITS		Initial denaturation: 95ºC – 6min
	X 35 cycles	Denaturation: 94ºC – 40s Annealing: 53ºC – 40s Extension: 72ºC – 1 min
		Final extension: 72ºC – 5 min
16S rDNA		Initial denaturation: 94ºC – 4min
	X 25 cycles	Denaturation: 94ºC – 30s Annealing: 50ºC – 30s Extension: 72ºC – 45s
		Final extension: 72ºC – 5min

To confirm the expected size and quality of PCR fragments - 4 µl of each amplification product was mixed with 2 µl of 6x loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 30% v/v glycerol) and separated by electrophoresis on a 1% agarose gel (Bioline, London, UK) in 1x TAE buffer at 90 volts and for approximately 45 minutes.

The agarose gel had ethidium bromide (10mg/ml), which is an intercalating agent commonly used in molecular biology to stain DNA. A molecular weight marker with 100bp Plus DNA Ladder (Gene ruler TM) was used as standard.

The PCR products were purified with the Illustra Exostar (GE HealthCare,USA) kit and according to the manufacturer's protocol.

3.3.4. Sanger Sequencing

The purified PCR products were sequenced using the ITS1 primers for eukaryotes and 16S_R2 for prokaryotes. The sequencing reaction consisted in a 10µl volume reaction with 2 µl Big Dye Terminator Kit v3.1 Cycle Sequencing (Applied Biosystems, USA), 2µl of the Big Dye Terminator v1.1 sequencing buffer, 30-50 ng of DNA, 3.2 pmol of primer and sterile milli-Q water. Sequencing reaction conditions are described in the Table III.

Table III: Sequencing reaction conditions.

Primers	Reaction condition	
ITS1		**Initial denaturation: 96ºC – 3min**
	X 25 cycles	**Denaturation: 96ºC – 10min** **Annealing: 53ºC – 5s** **Extension: 60ºC – 4min**
16S_R2		**Initial denaturation: 96ºC – 3min**
	X 25 cycles	**Denaturation: 96ºC – 10min** **Annealing: 50ºC – 5s** **Extension: 60ºC – 4min**

The products of this reaction were purified with the BigDye xTerminator Purification Kit (Applied Biosystems, USA) followed the manufacturer's instructions. Thereafter, samples were sequenced on 3500 Genetic Analyser sequencer (Applied Biosystems, USA).

The ChromasPro (Technelysium Pty Ltd) software was used to visualize the electropherograms and to edit the DNA sequences. Then the Genbank database (NCBI), through the nucleotide blast (Blastn), was used to find the closest match for each sequence based on the maximum identity in order to identify the isolates.

3.4. In vitro analyses for the secreening of biocontrol potential

3.4.1. Antagonistic activity

In vitro assays were performed to determine the biocontrol potential of bacteria and yeast isolates on the mycelial growth of eight fungal pathogens. For the analysis of the

antagonist activity two tests were applied – (1) general screening test (Figure 3A) and (2) co-culture (Figure 3B).

The first test allowed for the pre-selection of microorganisms with potential antagonist activity against fungal pathogens through the inhibition of mycelium growth. For this purpose, four different bacteria or yeast strains with 48/72h of growth were inoculated around the fungal pathogen (3mm of mycelium agar disk) with 7/15 days old and at 2,5 cm distance from the fungal (Figure 3A). This assay was performed in Petri dishes (9 cm) containing PDA medium and in triplicate. The plates were incubated at 22ºC for 15 days and for this test, no mycelial inhibition was calculated.

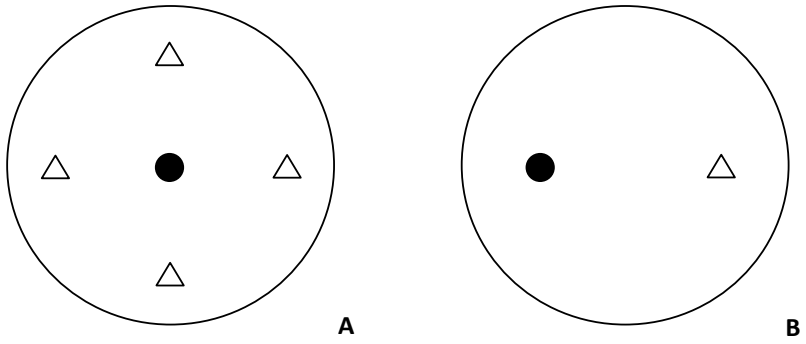

Figure 3: Scheme to evaluate the potential of antagonism.
A: Pre-screening test of potential antagonistic microorganism. B: Co-culture test.
△ Represents the bacterial and yeast isolate. ● Represents phytopathogen fungus.

The co-culture test (Figure 3B) allowed for the individual evaluation of the potential antagonist activity of each isolate towards fungal plant pathogenic (Pinto, 2011). The phytoprotectors were inoculated at 2.5 cm distance from the border of the plate, and in the opposite site, at the same distance, was inoculated the phytopathogen (Figure 3B). The plates were incubated under the same conditions as the first antagonism assay and in

triplicate. For this assay, the mycelial growth inhibition was calculated through the following formula:

$$\%\ \text{Micelial inhibition} = \frac{(Mb - Ma)}{Mb} \times\ 100$$

Where Mb corresponds to the free mycelium growth of plant pathogenic fungi and Ma corresponds to the mycelium growth of the fungal pathogen in the presence of the antagonist microorganism.

The values obtained from the mycelial inhibition growth were used to compare the power effect of the antagonistic microorganisms against fungal pathogens and the minimum significant difference ($\alpha = 0.05$) was analysed. The Mann Whitney U test was used to determine the minimum significant difference between the effect of antagonistic microorganisms in relation to the control (free growth of fungal pathogen), which the results failed to meet the assumption of normality. Data were analysed using SPSS® V17.0 (SPSS Inc., Chicago, IL, USA).

3.4.2. Siderophore production

The production of siderophores was analysed using the method of Chrome azurol S (CAS) described by Alexander and Zuberer, (1991) with slight modifications and only those microorganisms with significant antagonistic activity were tested. This medium which contains iron in limited quantities and wherein solutions composition are described in the Annex I, allows for the *in vitro* qualitative analysis of siderophores production through the observation of halos around bacterial colonies.

The production and diffusion of siderophores by microorganisms is responsible for a color change of culture medium, as a result of the removal of iron from the complex Fe - siderophore. The assay was performed according to the Figure 4 and plates were incubated for 10 days at 30°C in triplicate.

The color modification of the ternary complex CAS - Iron III – CTAB from blue to yellow halo around bacterial colonies indicate the production of siderophores (Silva-Stenico *et al.*, 2005).

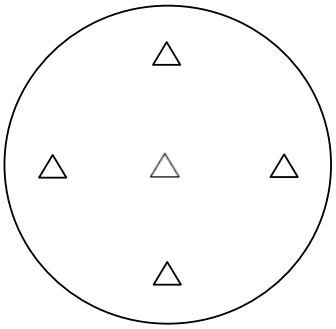

Figure 4: Scheme to evaluate the growth promoting properties.
Position of the phytoprotetors on the plate (△)

3.4.3. Phosphate solubilisation

The phosphate solubilisation by antagonistic isolates was qualitatively evaluated with Pikovskaya medium (Pinto, 2011) (Annex I). The assay was performed according to the Figure 4 and in triplicate. The plates were incubated for 10 days at 30 °C. Isolates with the capacity to solubilise phosphate (positive reactions) showed a halo or a translucent area around colonies. The diameter of the halo was measured and the solubilisation index was calculated using the following formula:

$$\text{Solubilization Index (SI)} = \frac{\text{Halo diameter (colony + halozone)(mm)}}{\text{colony diameter (mm)}}$$

Based on the solubilisation index, the isolates were classified as low (SI<2), intermediate (2≤SI<4) or high (SI≥4) (Marra *et al.,* 2011).

3.5. Effects of phytochemicals on antagonistic microorganisms

For this test, we have selected six isolates (as Fito_S127B, Fito_S247, Fito_F251, Fito_F289, Fito_F290 and Fito_S341) that simultaneously inhibited the mycelia growth of all phytopathogens tested, four yeast species (namely, Fito_F23, Fito_45, Fito_M113 and Fito_M141) and five isolates (Fito_M82A, Fito_F264, Fito_F271, Fito_F319 and

Fito_F350) that produce both siderophores and solubilize phosphate. These isolates were tested for their sensitive/tolerance towards trade formulates of 5 fungicides and 1 herbicide commonly used on vineyards from Bairrada region. Commercial formulates and active compounds of each phytochemical are reported in Table IV.

For each phytochemicals, three concentrations were prepared by adding stock phytochemical solutions, to PDA and 10 ml aliquots were poured immediately into Petri dishes.

For this test, isolates were grown overnight in PDB at 28ºC and 150 rpm, then a concentration of 10^6 to 10^8 cells/ml was inoculated in PDA plates containing different concentrations of the active element of phytochemical. For each phytochemical, four PDA plates were prepared: 1) no phytochemical (control), 2) half of the recommended dose, 3) recommended dose and 4) double of the recommended dose. For the inoculation, a liquid handling station (Sciclone ALH 3000 Workstation) equipped with a 96 pin-tool was used. The plates were incubated at 28 for 72h and each experiment was performed in triplicate.

Table IV: Phytochemical used in this study.

Commercial name	principal active elements	Recommended dose (µg/ml or µl/ml)	Diseases
Ridomil goldi combi	Folpet + Metalaxyl -M	2000	Mildew
Kocide 2000	Cooper hydroxide	2000	Mildew
Topaze	Penconazol	0,35	Powdery Mildew
Ridomil goldi combi + Topaze	Folpet + Matalaxyl – M + Penconazol	2000 + 0,35	Mildew + Powdery Mildew
Quadris max	Azoxistrobin + Folpet	1,5	Mildew + Black rot
Touchdown	Glyphosate	0,01	Weed

4 – Results and Discussions

4.1. Distribution of the isolated microbial communities

Over the 2011 and 2012 *Vitis vinifera* vegetative cycle, a total of 354 strains were isolated from soil, roots, stems, leaves, grapes and musts from symptomless and infected grape plants. From these, 214 isolates were bacteria or yeasts and 140 were identified as filamentous fungi. While most of the bacteria and yeasts were obtained from soil, must, and grape fruit, the filamentous fungi were mostly recovered from root, stems and petiole (Figure 5).

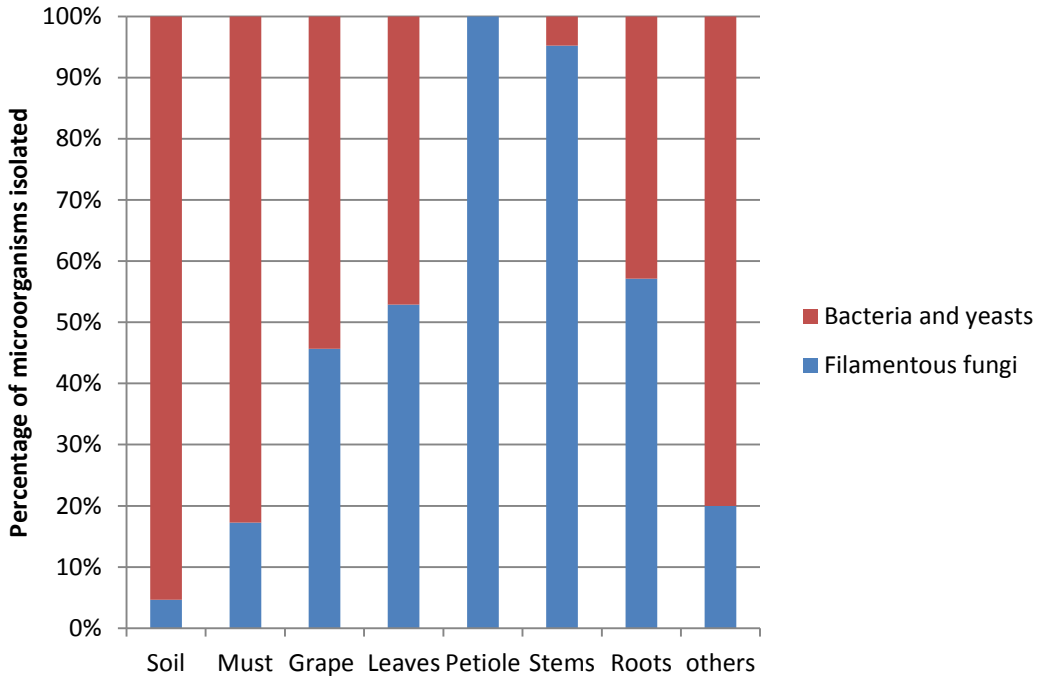

Figure 5: Diversity of the microbial community found in soil and grapevine.
Microbial community distribution in the different samples; others – isolates existing in the laboratory choose to the antagonism test.

Indeed, this finding is in line with the previous finding that plant pathogenic microorganisms attack the green parts of plants (leaves) and stems, and are responsible for infections occurring after fruit harvesting (Blodgett and Swart, 2002; Musetti *et al.*, 2005). These organs usually have a high nutrient content, favouring the fungi choice to infect these structures and to cause plant damage (Kaymak, 2010).

Regarding the bacterial population, they were mainly isolated from soil,which are known to be rich in exudates produced by plant roots (in particular the rhizosphere) making this restrict environment favourable for the nutrition of the bacterial community (Singh *et al.*, 2004; Mendes *et al.*, 2013).

The grape musts samples showed a higher percentage of bacteria and yeasts than filamentous fungi. The acidic pH as well as the high contents of sugar and alcohol are likely the main cause explaining the strong presence of these two groups of organisms(Barata *et al.*, 2012).

4.2. Diversity of isolated microbial communities

4.2.1. Phytoprotectors species and their spatial distribution

As referred in the previous chapter, only bacteria and yeasts showing antagonist capacity were identified through molecular analysis. A PCR was carried out to identify both bacteria, based on the amplification of the 16S rDNA with 16S_R2 and 16S_F2 primers and yeasts through the amplification of the ITS region with ITS1 and ITS4 primers (White *et al.*, 1990). The 16S primers amplified DNA fragments with 1600 bp (Figure 6A) and the ITS primers amplified DNA fragments from 600 bp (Figure 6B).

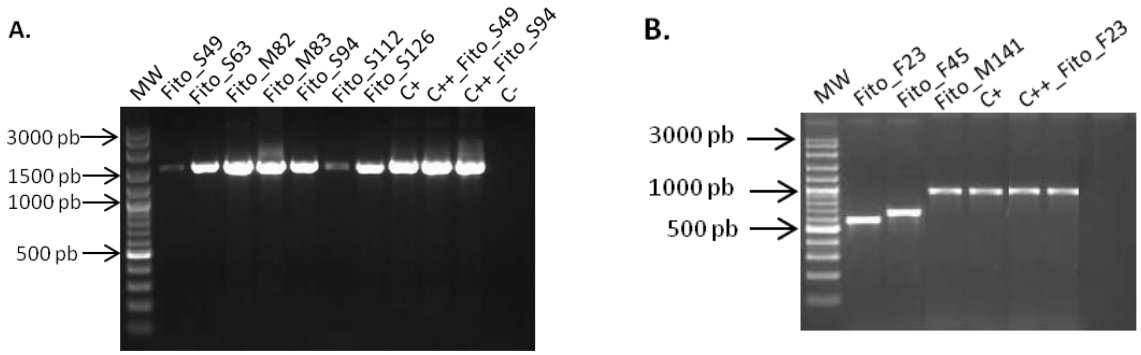

Figure 6: Ribossomal DNA amplification and ITS amplification.
Eletrophoretic separation of PCR products from the 16S (A) and ITS (B) rDNA amplification in 1% agarose gel. MW – Molecular weight marker with 100pb.

From the sequencing of the 72 isolates, 44 prokaryotic (61%) and 14 eukaryotic microorganisms (20%) were identified (Figure 7). A total of 14 isolates (19%) were not possible to identify due to problems related with DNA extraction or by ineffective PCR amplification (Figure 7).

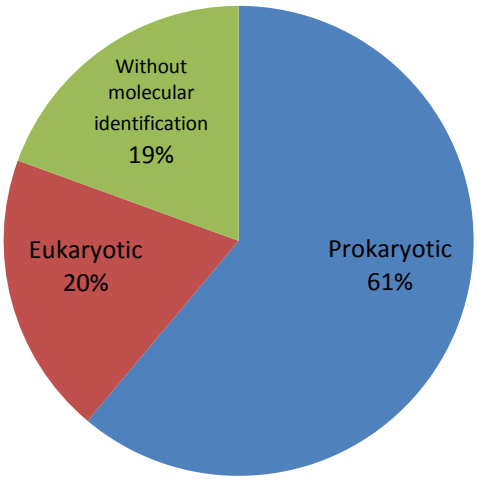

Figure 7: Diversity of microorganisms isolate.
Percentage of prokaryotic and eukaryotic communities identified.

The prokaryotic community was mostly dominated by bacteria of the genera *Bacillus* and *Streptomyces* (Figure 8). The species belonging to the *Bacillus* genus were *Bacillus amyloliquefaciens* (3 isolates), *Bacillus cereus* (6 isolates), *Bacillus methylotrophicus* (8 isolates), *Bacillus sp.* (7 isolates), *Bacillus subtilis* (3 isolates), *Bacillus tequilensis* (3

isolates) and *Bacillus vallismortis* (1 isolate) (Figure 8). From the *Streptomyces* genus, we have identified the *Streptomyces chartreusis and Streptomyces coelicolor* species. These bacteria are known to reside naturally in soils, which justifies our finding (

Figure 9). Accordingly to the literature, these microorganisms are commonly isolated from grape rhizosphere soils (Karagoz *et al.*, 2012). Indeed, they are involved in some important processes in soil such as decomposition of organic matter, soil structure formation, and carbon and other elements (nitrogen, sulphur, phosphorous) cycling (Crawford *et al.*, 1993; Dardanelli *et al.*, 2010).

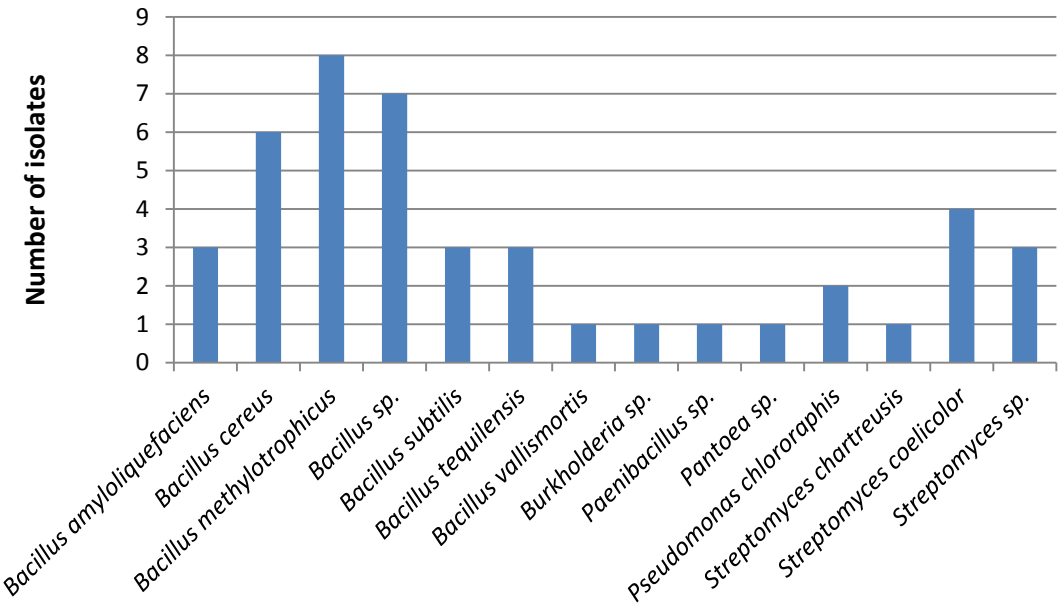

Figure 8: Abundance of the bacterial species identified.

Also, the genera *Burkholderia*, *Pantoea* and *Paenibacillus* were also found in our samples. However, it was not possible to distinguish different species in each one of these genera, as using BLAST against the Genbank, thus it were be necessary to further sequencing these samples and to target different genes.

Among the eukaryotic community, the most dominant genus identified was *Aureobasidium*, represented only by the *Aureobasidium pullulans* isolated from leaves and grape (

Figure 9). This species is a dimorphic ascomycete fungus, commonly known as black-yeast which has been widely reported both as an epiphyte and as an endophyte microorganism in grapevine (Pancher *et al.*, 2012). The genus *Metschnikowia*, represented by the species *Metschnikowia pulcherrima*, was also identified and isolated from grape and must samples (Figure 10). This fermentative ascomycetous is an organism commonly isolated from wine grapes at the harvesting time (Prakitchaiwattana *et al.*, 2004) and further on at the beginning of fermentation stage (Barata *et al.*, 2012).

Figure 9: Distribution of phytoprotectors according to their isolation source.
NID: Not identified.

The species *Cryptococcus magnus*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae* and *Ustilago cynodontis* were also isolated although at lower frequencies (Figure 10). *Cryptococcus magnus* is a typical leave-colonizing microorganism (Čadež *et al.*, 2010) and was isolated from must samples, whereas *Hanseniaspora uvarum*, an apiculated yeast, was isolated from grapes (Sabate *et al.*, 2002).

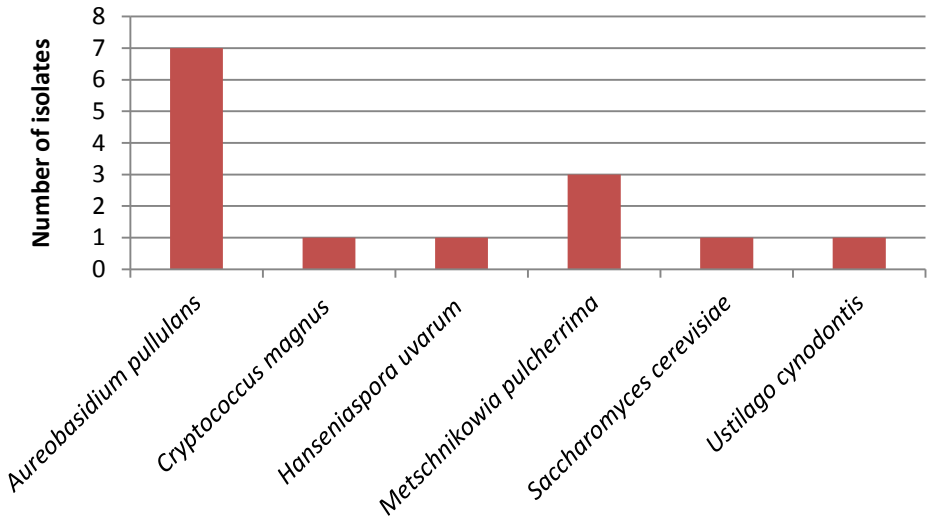

Figure 10: Abundance of yeast species identified from soil and grapevine parts.

These results are in accordance with previous studies, where these genera were isolated from soil and vine (Crawford *et al.*, 1993; Trotel-Aziz *et al.*, 2008; Bulgari *et al.*, 2009; Loqman *et al.*, 2009; Karagoz *et al.*, 2012). However, some species of the genera

Arthrobacter, Azoarcus, *Azospirillum*, *Klebsiella*, and *Serratia* that are often considered antagonists and plant growth promoters (Dardanelli *et al.*, 2010) were not identified in this study.

4.2.2. Filamentous fungi species and their spatial distribution

The diversity of the filamentous fungi was investigated by the sequence analysis of the ITS region (Figure 11). From the 140 filamentous fungi isolated, 112 were identified. Among those, *Alternaria* sp. were the most abundant, accounting for 67% of the total microorganisms isolated from leaves (Fig. 12).

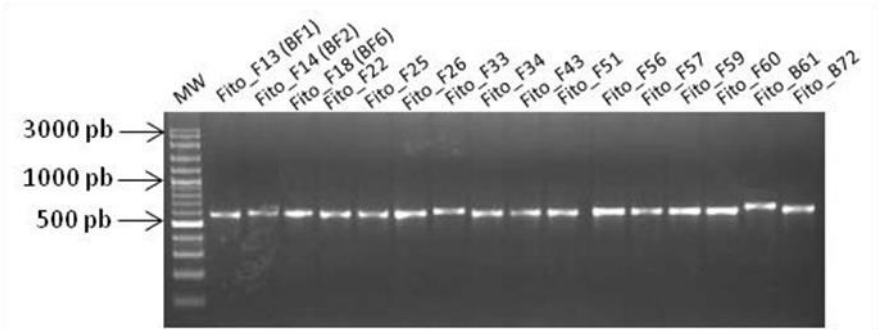

Figure 11: ITS region amplification.
Separation of the PCR products of the ITS region using the primers ITS1 and ITS4 on 1% agarose gel.

The specie *Alternaria alternata* was the most abundant species of this genus (Figure 12). Previous reports have shown that *Alternaria* has been associated with endophyte microorganisms and latent pathogens of grapevine (Blodgett and Swart, 2002; Musetti *et al.*, 2006) and recent reports indicated that *Alternaria alternata* complex was the most abundant group found in grapevine (Polizzotto *et al.*, 2012). Other reports showed that a species of this genus is responsible for the leaf spot diseases, characterized by the appearance of lesions on leaves leading to the collapse and cell death in *Amaranthus*

hybridus (Blodgett and Swart, 2002). Interestingly, and despite being considered a phytopathogen, some *A. alternata* strains have been demonstrated to inhibit *Plasmopara vitcola* sporulation in grapevine leaves (Musetti *et al.*, 2006).

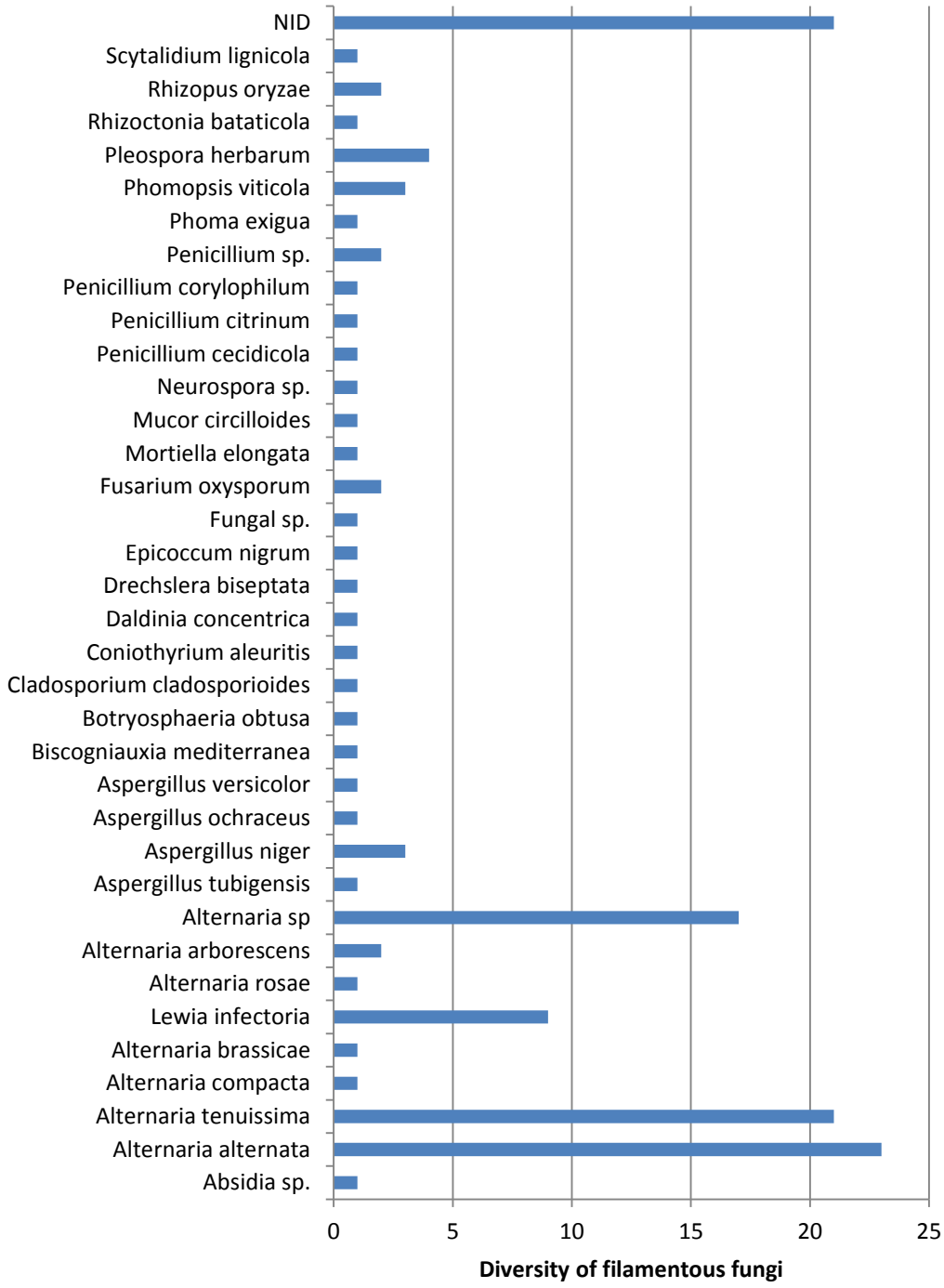

Figure 12: Microbial diversity and abundance of the filamentous fungi identified from soil and grapevine.

NID: Not identified.

Other fungal genera identified in our samples were *Penicillium* sp. namely, the species *P. cecidicola, P. citrinum, P. corylophylu; a*nd *Aspergillus,* including *A. tubigensis, A. niger, A. ochraceus* and *A. versicolor* (Figure 12). Both genera are quite relevant because they are responsible for ocratoxin_A (OTA) production, a toxic secondary metabolite that contaminates red wines (Sage *et al.*, 2002; Pinto *et al.*, 2014). Therefore, they have negative impact on the final quality of wine.

Other fungi, also responsible for *Vitis vinifera* diseases, were also identified. Among them were *Botryosphaeria obtusa,* a canker causing agent responsible for the black dead arm diseases in grapevine (Urbez-Torres *et al.*, 2010), *Pleospora herbarum,* the causal agent of leaf spots in the leguminous alfalfa and red clover (Sadowsky *et al.*, 2007), *Drechslera biseptata* responsible for root rot (Abu-Taleb *et al.*, 2011) and *Scytalidium lignicola,* a phytopathogen responsible for the wilt diseases on citrus tree (Oren *et al.*, 2001).

It is worthwhile to notice that, we did not identify over our samples the fungal pathogens *Plasmopara viticola* and *Uncinula necator*, responsible for downy mildew and powdery mildew, respectively. In fact, these species are obligate parasites, which means that it is not to possible isolate them through a culture dependent approach.

4.3. In vitro analyses of the biocontrol potential

4.3.1. Antagonistic activity

A total of 214 isolates listed in the group of bacteria and yeast were screened for their antagonistic potential to control/ inhibit the mycelial growth of 8 fungal phytopathogens, through the *in vitro* antagonistic tests (Figure 13).

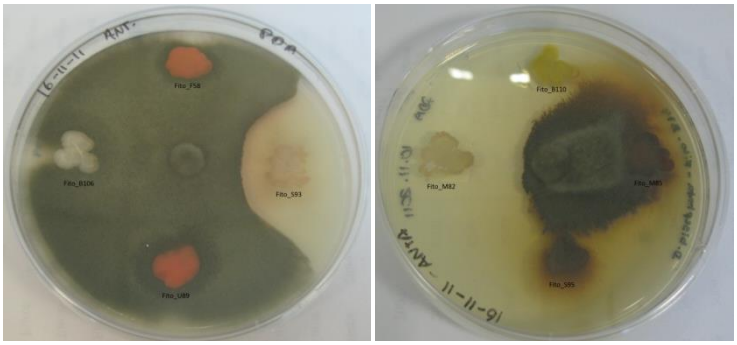

Figure 13: First antagonism test (pre-screening) with the phytopathogen *Drechslera biseptata.*

In the first pre-screenning test, 67 isolates showed to be antagonistic against *Botryosphaeria obtusa*. The species with more isolates with potential antagonistic activiy were *Pleospora herbarum*, *Lewia infectoria* and *Drechslera biseptata* with 109, 106 and 98 isolates, respectively. In contrast, the species with the lowest number of isolates with potential antagonist activities were *Alternaria compacta* (46 strains), *A. brassicae* (46 isolates) *A. alternata* (73 isolates), and *Scytalidium lignicola* (79 isolates) (Figure 14).

The isolates that inhibited phytopathogen's mycelial in the first antagonism assay were further selected to be tested in the co-culture assay. The results showed that 72 isolates had antagonistic activity. According to our results, the isolates Fito_S127B (*Streptomyces* sp.), Fito_S247 and Fito_S341 (*Bacillus subtilis*), Fito_F251 (not identified), Fito_F289 (*Bacillus methylotrophicus*), and Fito_F290 (*Bacillus* sp.) showed the highest antagonistic effect against the 8 fungal pathogens tested. Among these, best results of inhibition growth were observed with Fito_S127B (*Streptomyces* sp.) (Figure 15).

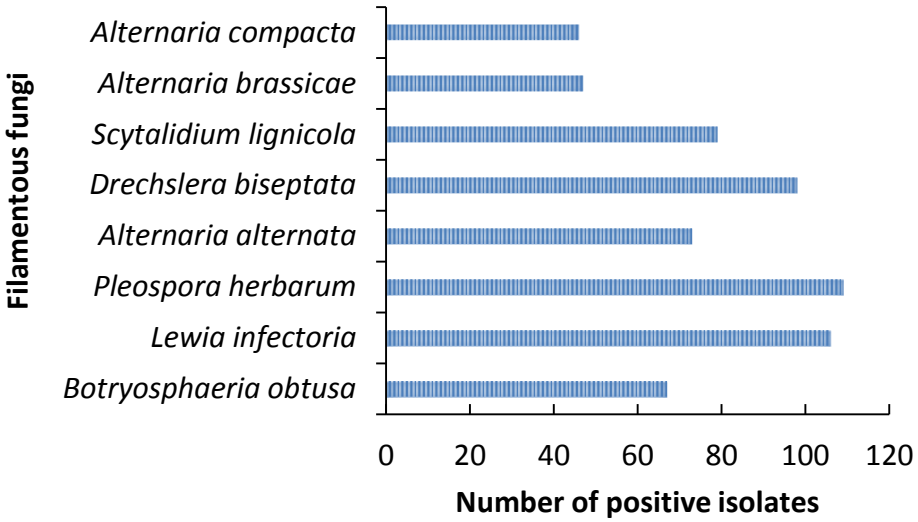

Figure 14: Effectiveness of the 214 isolates in inhibiting the mycelium growth of the phytopatogens selected in the first antagonism test.

The species *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus methylotrophicus*, *Bacillus subtilis*, *Bacillus tequilensis* and *Bacillus vallismortis* correspond to the species belonging to *Bacillus* genus with significant antagonistic activity ($\rho<0.05$).

Bacillus species are well known for their antagonistic activity and have been considered as good candidates as biological control agents. Due to the production of secondary metabolites with antifungal properties (Wulff *et al.*, 2002).

Although these antifungal compounds were not characterized in this study, different antibiotics (*e.g.* iturin, fengycin, bacillopeptins and surfactin) and some hydrolytic enzymes (*e.g.* such as proteases, chitinases, cellulases, amylases and glucanases) have been described as being produced by the *Bacillus* sp. and displaying a strong antifungal activity against a wide range of phytopathogens (Quan *et al.*, 2010). Wulff and collaborators (2002) stated that these metabolites not only antagonize the pathogens but also trigger host defense responses thus increasing plant protection (Wulff *et al.*, 2002).

Species of *Bacillus* are also very attractive as potential inoculants since they can be easily formulated due to endospore formation, which can survive for prolonged periods in industrial formulations (Figueiredo *et al.*, 2010). This explains the higher availability of biopesticides and biofertilizers products based on *Bacillus* (Berg, 2009; Figueiredo *et al.*, 2010).

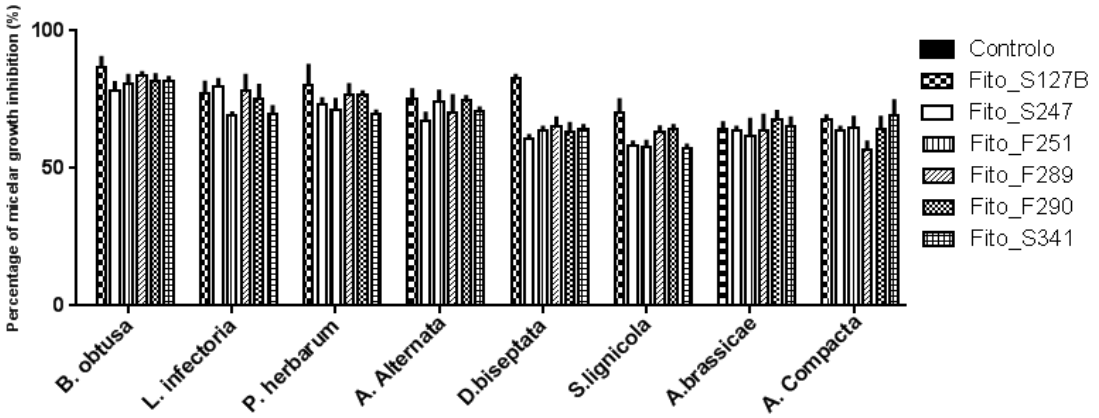

Figure 15: Isolates with antagonistic activity showed a mycelial growth inhibition >50% against the 8 fungal phytopathogens tested.
For each isolate, the mean and the standard deviation of the three replicates used is considered.

In this study, the isolates from the species *Streptomyces chartreussis* and *S. coelicolor*, and *Streptomyces* sp. Have also showed to be effective on the limitation of mycelium growth. This might be explained by the ability of these isolates to produce chitinases (Saito *et al.*, 1999; Nazari *et al.*, 2013), siderophores (Tierrafría *et al.*, 2011) or other antifungal substances to the surrounding area. The inhibitory role of *Streptomyces* sp. in *in vitro* assays against other phytopathogens such as *Fusarium* sp., *Alternaria* sp., *Curvularia* sp., *Colletrotrichum* sp. and *Aspergillus niger* has been reported by other authors(Evangelista-Martinez, 2014).

According to other studies, the mycelium growth of the phytopathogens as *Fusarium* sp., *Alternaria* sp., *Curvularia* sp., *Colletrotrichum* sp. and *Aspergillus niger* was inhibited by *Streptomyces* species in *in vitro* tests (co-culture) (Evangelista-Martinez, 2014).

The bacterial isolates belonging to *Burkholderia* sp., *Pseudomonas chlororaphis* and *Paenibacillus* sp. also showed antagonistic activities against some fungal phytopathogens

selected, which is in line of the reported production of several secondary metabolites with antifungal properties (such as antibiotics, alkaloids and siderophore) (Trotel-Aziz *et al.*, 2008; Quan *et al.*, 2010; los Santos-Villalobos *et al.*, 2012).

Other beneficial bacteria such as *Pantoea* sp. have been reported as effiective epiphytic biocontrol agents. In this study, the isolate belonging to *Pantoea* sp. showed a significant inhibition of the mycelial growth of the phytopathogen *Drechslera biseptata*. Previous studies reported the isolation of microorganisms belonging to this genus and their potential as biocontrol agents against a wide spectrum of plant phytopathogens (Trotel-Aziz *et al.*, 2008; Bulgari *et al.*, 2009).

In addition to these isolates, the yeasts *Aureobasidium pullulans*, *Cryptococcus magnus*, *Hanseniapora uvarum*, *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae* and *Ustilago cynodontis*, also showed antagonist activity.

Several possible biological mechanisms that explain the biocontrol activity have been suggested, but the competition for space and nutrients has been widely suggest as the major mode of action of the yeasts (Saravanakumar *et al.*, 2009). Indeed, *A. pullulans* colonizes leaves (Pinto *et al.*, 2014) and fruits, explaining its successful use in postharvesting biocontrol and against foliar diseases (Schena *et al.*, 2003; Schmid *et al.*, 2011). Being naturally adapted to these niches, they are able to effectively colonize and compete for nutrients and space against opportunistic microorganisms (El-Tarabily and Sivasithamparam, 2006).

Recently, the yeasts *Saccharomyces cerevisiae*, *Cryptococcus magnus* and *Metschnikowia pulcherrima*, commonly associated with microbiome of grapefruit and wine fermentation (Barata *et al.*, 2012), have been reported as effective biocontrol agents against the phytopathogens of the genus *Penicillium* and *Aspergillus*, both producers of mycotoxins in wheat after harvest (Petersson and Schnürer, 1995). Similar observations were obtained for *Monilinia*, which is responsible to the most important postharvest diseases in nectarines and peaches (Janisiewicz *et al.*, 2010; Zhang *et al.*, 2010).

Interestingly, some species may have a dual role. This is the case of *Ustilago cynodontis*, referred as a phytopathogen in grapevine (Pinto *et al.*, 2014), but showing

activity against the phytopathogens *Lewia infectoria* and *Pleospora herbarum* in our assays.

The different metabolic characteristics of the antagonistic isolates developed different behaviours against phytopathogen microorganisms. When the phytopathogenic fungi were grown freely in Petri dishes (in the absence of the antagonist) they promptly occupied the entire surface of the culture medium and formed spores (Figure 16A). However, in the presence of some microbial antagonists the fungal mycelium was clearly inhibited and a clear zones could be seen (Figure 16B –Figure 16I).

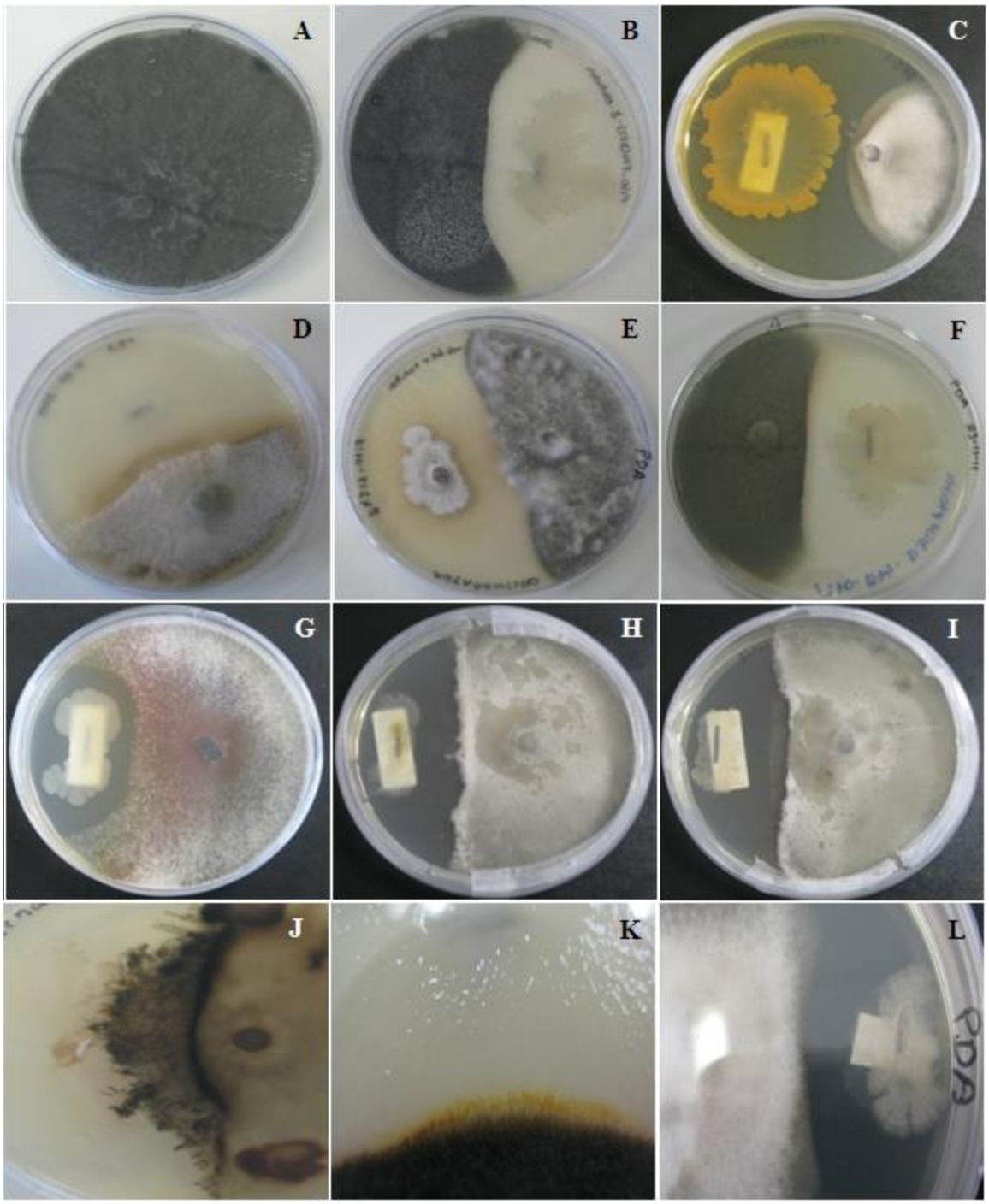

Figure 16: Different aspects of the co-culture assays.
A: Control showing the free growth of Botryosphaeria obtusa. B: Botryosphaeria obtusa and Bacillus amyloliquefaciens (Fito_S122). C: Lewia infectoria and Pseudomonas chlororaphis (Fito_S227). D: Pleospora herbarum and Bacillus cereus (Fito_S124). E: Alternaria alternata and Streptomyces sp. (Fito_S127B). F: Drechslera biseptata and Bacillus sp. (Fito_M83). G: Scytalidium lignicola and Bacillus methylotrophicus (Fito_S230). H: Alternaria brassicae and Bacillus tequilensis (Fito_F224). I: Alternaria compacta and Bacillus amyloliquefaciens (Fito_F317). J: Alternaria alternata and Burkholderia sp. (Fito_S63). K: Drechslera biseptata and Bacillus cereus (Fito_F7). L: Lewia infectoria and Bacillus cereus.

The results also indicated that during the co-culture test, most of the interactions between antagonistic and phytopathogens did not involve any physical contact between both microorganisms. However, whenever a contact between both microorganisms occurred, a destruction of fungal mycelia was observed (Figure 16J and Figure 16K). According to this observation, it is reasonable to assume that the inhibition of fungal growth may be due to antifungal substances excreted into the culture medium by the antagonist microorganisms. In fact, this not only interfered with the normal process of mycelial growth of fungi but also with the development and maturation of the spores (Ferreira *et al.*, 1991) (Figure 16L).

Among all the antagonistic microorganisms, 13 isolates were not molecularly identified because not enough DNA was extracted and/or a successful amplification could not be achieved.

4.3.2. Siderophore production and phosphate solubilisation by phytoprotectors

The capacity of the microorganisms to produce siderophores was assessed on CAS medium and phosphate solubilisation on Pikovskaya medium. The reactions, positive or negative, were defined according to the halo formed around colonies. Thus, and as referred on material and methods, the isolates in which siderophore production occurred presented an orange/yellow halo in the CAS medium and the isolates with capacity to solubilise phosphate presented a clear halo around the colonies, in Pikovskaya medium supplemented with calcium phosphate (Figure 17).

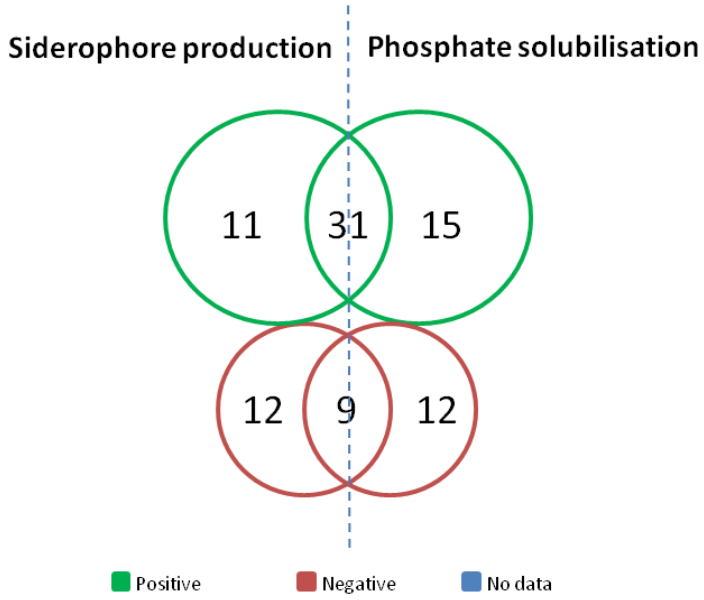

Figure 17: Total of isolates analyzed for their growth promoting ability.

The 72 isolates with antagonist activity equal or higher than 50% of the mycelium inhibition growth were analysed for their ability to produce siderophores and solubilise phosphate. A total of 31 isolates were able to simultaneously produce siderophores and solubilize phosphate. Whereas, 11 isolates only showed capacity to produced siderophores and 15 isolates to solubilise phosphate, and 10 isolates have not grown, thus no data was acquires.

The Table V shows the most promising phytoprotector isolates obtained in this study. Among the yeasts, *Aureobasidium pullulans* (Fito_F23) and *Saccharomyces cerevisiae* (Fito_M141) showed positive reactions both for the siderophore productions and phosphate solubilisation. Interestingly, *Saccharomyces cerevisiae per si* lacks the ability to produced siderophores (Haas, 2003), but when near other species, can utilize the exogenous siderophores produces by another species and then used them to adquires iron. Thus, explaining our observations. Six isolates from *Bacillus* showed also to be able to produce siderophores and to solubilise phosphate. Among the B*acillus* genus, two *Bacillus subtilis* were positive for these reactions thus sustaining earlier observations, which of siderophore production in response to iron deprivation (May *et al.*, 2001; Hu and Xu, 2011).

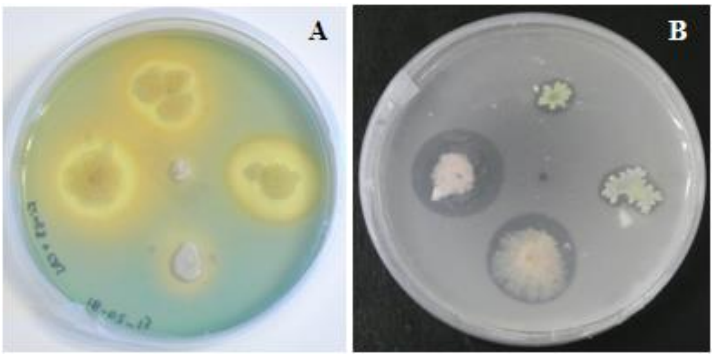

Figure 18: Qualitative analysis of the growth promoting characteristics of the phytoprotector.
A: Qualitative detection of siderophore production on CAS medium. B: Solubilisation halo of the inorganic phosphate in Pikovskaya medium.

Thirteen isolates out of the fifteen tested showed ability to solubilise phosphate. The phosphate solubilisation index (SI) of the different isolates varied from low to intermediate using a Pikovskaya medium. The highest SI among all the microorganisms analysed in this work was obtained with *Saccharomyces cerevisiae.*

The isolate *Streptomyces* sp. (Fito_S127B), which presented the highest percentage of mycelial inhibition growth, did not show the ability neither to produce siderophores nor to solubilise phosphate, under *in vitro* conditions. However, this isolate showed a significant antagonism against the agent associated with canker and declining in vines - *Botryosphaeria obtusa.* Therefore this isolate can be regarded as a potential biological control agent, but not as a plant growth promoter.

Table V: The most promising phytoprotector and their growth promoting ability

Strains	Species	Siderophore	Phosphate solubilisation	
		CAS medium	Pikovskaya medium	SI (mm)
Fito_F23	*Aureobasidium pullulans*	+	+	WD
Fito_F45	*Cryptococcus magnus*	-	+	1,17
Fito_M82A	*Bacillus vallismortis*	-	++	WD
Fito_M113	*Metschnikowia pulcherrima*	-	+	1,5
Fito_S127B	*Streptomyces* sp.	-	-	WD
Fito_M141	*Saccharomyces cerevisiae*	+	+	2,06 ± 0,12
Fito_S247	*Bacillus subtilis*	+	+	1,3 ± 0,12
Fito_F251	NID	+	+	1,3 ± 0,12
Fito_F264	*Bacillus subtilis*	+	+	1,17 ± 0,17
Fito_R271	*Bacillus tequilensis*	-	+	1,23 ± 0,22
Fito_F289	*Bacillus methylotrophicus*	+	-	1,05 ± 0,08
Fito_F290	*Bacillus* sp.	+	+	GNS
Fito_F319	*Bacillus* sp.	+	+	1
Fito_S341	*Bacillus subtilis*	+	+	1,10 ± 0,08
Fito_F350	*Bacillus* sp.	+	+	1,16 ± 0,17

Values indicate mean of three replicate. NID: Not identified; CAS: Chrome azurol S agar; SI: Solubilisation index; WD: Without data; GNS: Grew and did not solubilize (+) positive; (-) Negative reactions.

4.4. Effects of phytochemical in the performance of phytoprotectors microorganism

The Figure 19 and Figure 20 showed the compatibility of the identified potential phytoprotectors towards common fungicides *in vitro*. Generally, all the bacteria and yeasts tested proved to be very sensitive to the fungicides Ridomil (Figure 19B), Topaze (Figure 19D), a mixture of Ridomil and Topaze (Figure 20E) and Quadris max (Figure 20F) at all doses. Interestingly, while bacteria was very sensitivy to Touchdown at all doses, the yeasts were resistance to this herbicide (Figure 20G).

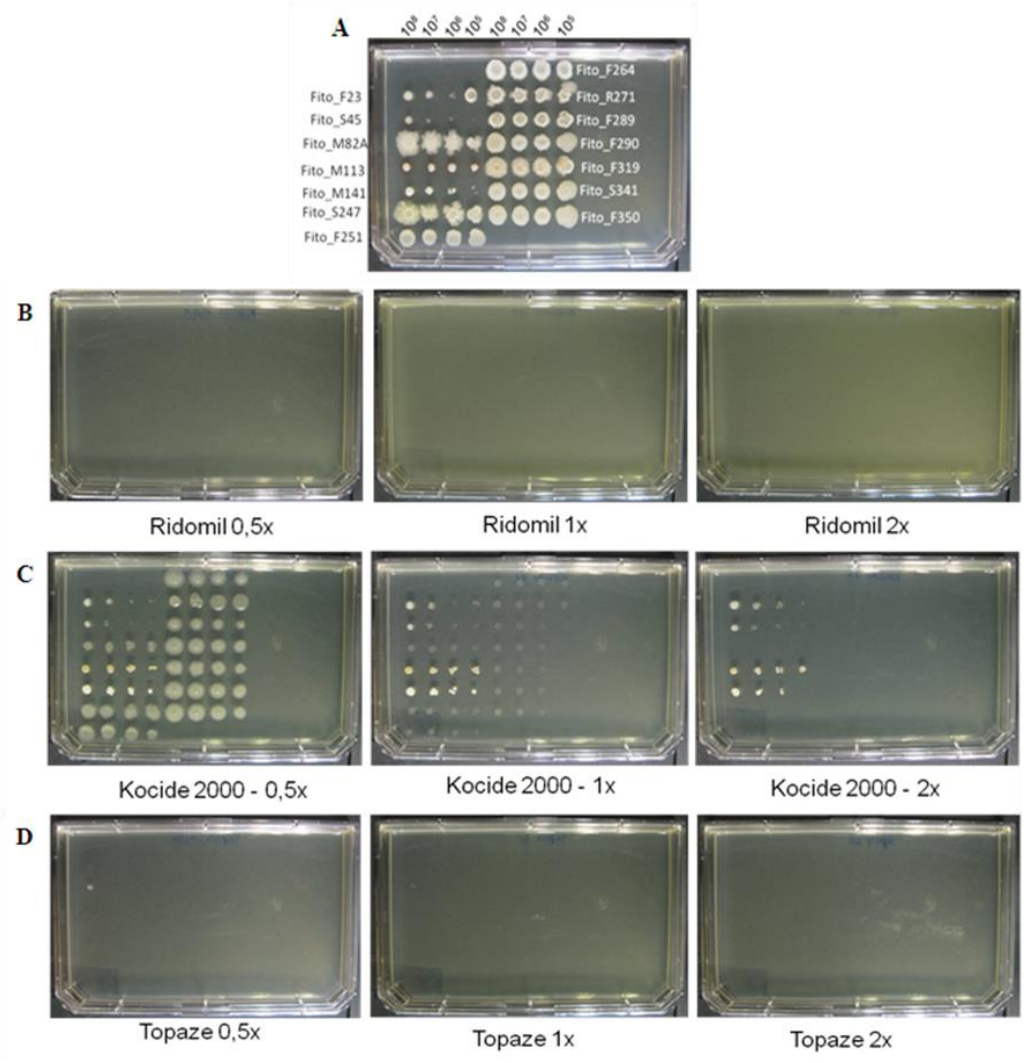

Figure 19: Growth of isolates on PDA supplemented with different concentrations of pesticides.
A – Control Plate. B – PDA supplemented with Ridomil on different concentrations. C - PDA supplemented with Kocide 2000 on different concentrations. D - PDA supplemented with Topaze on different concentrations.

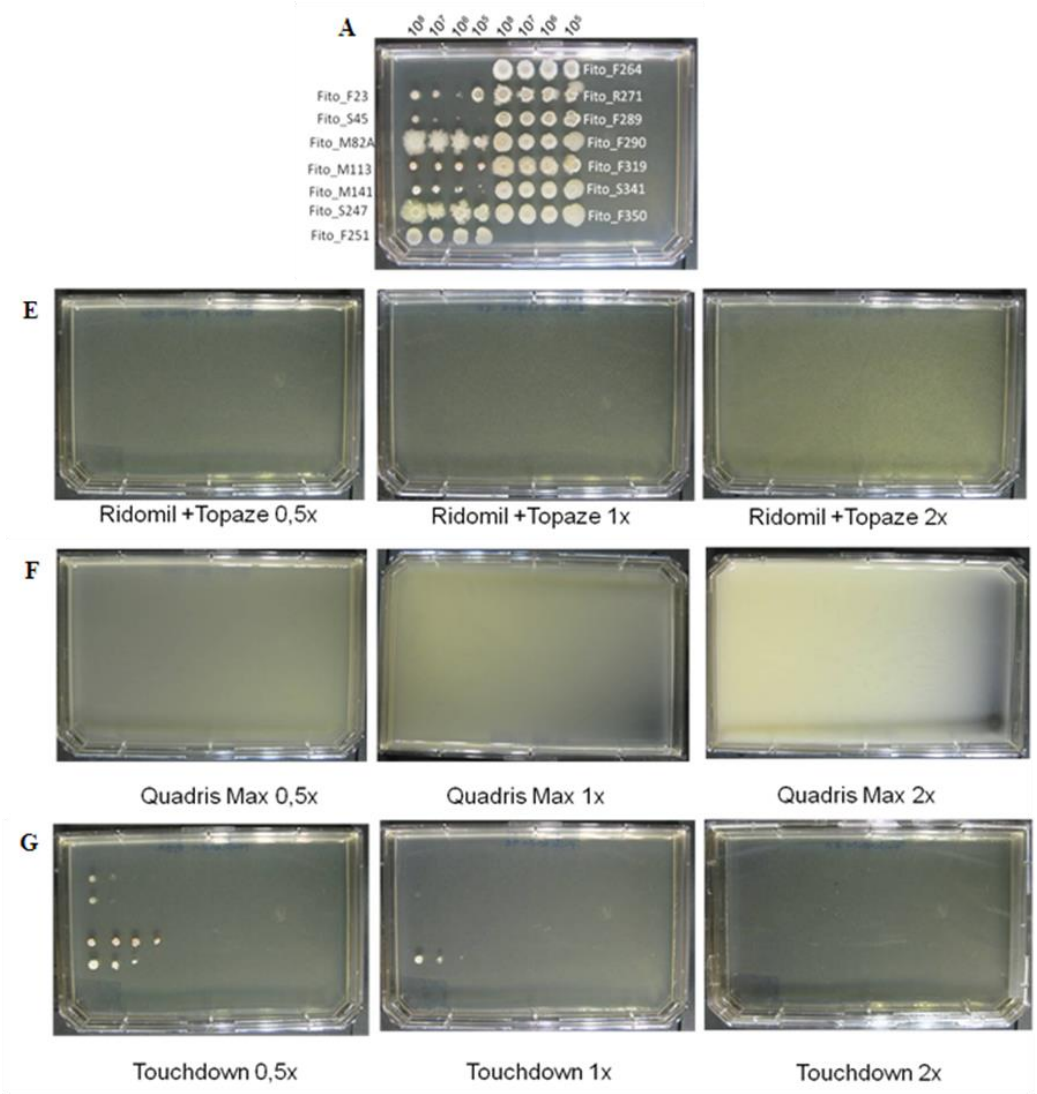

Figure 20: Growth of isolates on PDA supplemented with different concentrations of pesticides and herbicide.

A – Control Plate. B – PDA supplemented with Ridomil + Topaze on different concentrations. C - PDA supplemented with Quadris Max on different concentrations. D - PDA supplemented with Touchdown on different concentrations.

The selective effect of various fungicides on these bacteria and yeasts was observed by numerous researchers (Cervantes and Gutierrez-Corona, 1994; Lima *et al.*, 2006; Čadež *et al.*, 2010; Komarek *et al.*, 2010). According, Comitini and Ciani (2008), the fungicides directly applied on grapevine result in a dramatic reduction of yeast populations on grape (Comitini and Ciani, 2008).

The phenylamide fungicide, metalaxyl and protectant type fungicides, folpet, both are the active compounds of the fungicide Ridomil Goldi Combi is used as a foliar spray against fungal pathogens which cause mildew in grapevine (Monkiedje and Spiteller, 2005). The metalaxyl fungicides affect nucleic acids synthesis by inhibiting the activity of the RNA polymerase I systems (Yang *et al.*, 2011), while folpet inhibits normal cell division (Gisi and Sierotzki, 2008). Such effects on the cell's biochemistry can explain the lack of growth on the PDA supplemented with this fungicide. Indeed, such effect of metalaxyl and folpet on bacteria and yeasts was also observed by Moulas et al (2013), who demonstrated that the fungicide metalaxyl exerted a mild effects on the fungal and bacterial communities in pepper plants (Moulas *et al.*, 2013). Moreover, Arce et al, (2010) reported the induction of gene mutations on bacteria and yeasts in the presence of folpet (Arce *et al.*, 2010).

The sensitivity or resistance to copper (Kocide 2000) was found to vary between bacteria and yeast species. For example, *Aureobasidium pullulans* (Fito_F23), *Cryptococcus magnus* (Fito_F45), *Metschnikowia pulcherrima* (Fito_M113) and *Saccharomyces cerevisiae* (Fito_M141) demonstrated a high level of resistance with increasing of copper concentration which may be linked to different mechanisms.

In contrast, the bacteria showed to be sensity to copper (Kocide 2000) (**Erro! A origem da referência não foi encontrada.**) which consistent with the results reported by some authors including Martins et al (2012). In their study, they analyse the densities of the populations on grape berry surface, and showed a negative correlation between copper concentrations and cell densities, providing a clear evidence that the copper inhibited bacteria communities (Martins *et al.*, 2012).

Copper based fungicides are treatments commonly applied to control fungal diseases such as downy mildew, caused by *Plasmopara viticola* (Komarek *et al.*, 2010). Martins and collaborators (2012), showed that the increase of copper concentration affect the number and variability of the microbial communities present in vineyard (Martins *et al.*, 2012), which is in accordance with our results (Figure 19C). Other studies have also demonstrated both the sensity and the resistance of these yeasts to copper (Mortimer, 2000).

Interestingly, all the tested isolates are able to grow in presence of copper hydroxide (Kocide 2000), thus suggesting that these isolates could be successfully integrated with this fungicide in a field situations, with a minimal effect on the biocontrol agents.

When the bacteria and yeast was grown in PDA medium supplemented with penconazole (Topaze) and a mixture of metalaxyl, folpet and penconazole (Ridomil and Topaze) it was observable a repressive effect on growth of the isolates (Figure 19D and Figure 20E). Recently Jawich et al (2009), showed that penconazole was the most toxic fungicide for *Saccharomyces cerevisiae* and *Metchsnikowia pulcherrima,* affecting they growth and fermentation kinetics (Jawich *et al.*, 2006).

The Quadris Max is a mixture of azoxystrobin and folpet, possesses broad spectrum systemic activity against the four major classes of pathogenic (Adetutu *et al.*, 2008). These fungicides inhibit mitochondrial respiration by preventing electron transfer from cytochrome *b* to *c* and inhibiting energy production via oxidative phosphorylations. This inhibition of ATP synthesis then results in the death of the microorganism. Since azoxystrobin functions by inhibiting mitochondrial respiration, it is expected not have a direct effect on bacteria. However, in our study this fungicide does have an effect on bacteria, thus we can conclude that the folpet in the formulations affected the bacteria and yeasts.

We also observes that the yeasts isolates Fito_F23, Fito_45, Fito_M113 and Fito_M141 were more resistant to the phytochemicals than bacteria. In particular in medium supplemented with glyphosate (Touchdown) this discrepancy is notorious. The glyphosate (Touchdown) is a broad-spectrum metal chelating herbicide that inhibits the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS), which is necessary for the synthesis of aromatic amino acids in bacteria and fungi (Ratcliff *et al.*, 2006; Zobiole *et al.*, 2010). The toxicity effect of the glyphosate has been attributed to the inability of the organisms to synthesize aromatic amino acids.

According to Ratcliff et al (2006) the herbicide glyphosate has a benign affect on microbial community structure when applied at the recommended field rate, and produces a non-specific, short-term stimulation of bacteria at the high concentration (Ratcliff *et al.*,

2006). However, we have observed the opposite effect, in the recommended field rate (Touchdown 1x), where only the yeast *Saccharomyces cerevisiae* (Fito_M141) grew (Figure 20G).

In general, the in vitro experiment showed that applications of fungicides in any concentrations, even the lowest, reduced the phytoprotectors growth.

5 – Conclusions and future perspectives

The complexity of the interactions involved in biological control and the better knowledge of the genetic and metabolic characteristics of phytoprotectors remains a powerful resource to unveil the mechanisms involved in plant pathogenic control and plant growth promotion.

The increasing interest for microorganisms possessing phytoprotector characteristics and showing potential to induce plant growth lead us to use a combination of molecular and biochemical techniques to analyse the microbial community associated with *Vitis vinifera*. Some species representing putative antagonists were identified belonging to the genera *Aureobasidium*, *Bacillus*, *Cryptococcus, Metschnikowia, Pantoea*, *Paenibacillus*, *Pseudomonas*, *Streptomyces,* and *Saccharomyces,* . These yeasts species are considered as belonging to the flora of the vineyard and are involved in wine fermentation. Actually, these yeasts are involved in the biocontrol of postharvest diseases of plant products, especially fruit (Schena *et al.*, 2003). Also, the species belonging to the genera *Bacillus*, *Pantoea*, *Streptomyces* and *Pseudomonas* have been often recognised as potential biocontrol agents. For this reason, they have already been marketed in some countries, such as biofertilizers and biopesticides (Bailey *et al.*, 2010; Figueiredo *et al.*, 2010; Kaymak, 2010).

The most promising isolates with potential to be used as phytoprotectors, were the isolates of *Aureobasidium pullulans*, *Bacillus subtilis, Bacillus tequilensis, Bacillus methylotrophicus, Bacillus vallismortis, Cryptococcus magnus, Metschnikowia pulcherrima,* and *Saccharomyces cerevisiae*. They not only showed a significant inhibition of mycelia growth ($\geq 50\%$), but also showed positive reactions to the phosphate solubilization and production of siderophores.

Furthermore, our results clearly revealed a strong impact of the agrochemicals tested on phytoprotetors, even when tested at low concentrations, highlighting the importance of the screening and studing of the biological control agents. Further research is required to explore the effect of these agrochemicals in the solubilization of phosphate and producing of siderophores. Therefore, the isolates selected in this study could be used for future *in vivo* experiments to assess their ability to contribute to increased grapevine productivity.

In general, the combination of all these analyzes will allow to fully exploit the potential of these antagonistic microorganisms, thereby improving the knowledge of the interactions between plant pathogenic, phytoprotector microorganisms and *Vitis vinifera*. This work proved to be quite promising for the knowledge of potential biological control agents and will contribute, in the near future, to the development of sustainable and organic viticulture.

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7 - Annexes

Annex I: Culture medium

i. Potato dextrose agar medium (PDA)

- Potato dextrose agar (PDA): 42g/L

Add the component to distilled water and autoclaving for 15 min at 121ºC.

ii. Yeast extract peptone dextrose medium (YEPD)

- Yeast extract: 10g/L
- Dextrose: 20g/L
- Peptone: 20g/L
- Agar: 20g/L

All the components were added to distilled water and autoclaving for 15 min at 121ºC.

iii. Potato dextrose Broth medium (PDB)

- Potato dextrose broth (PDB): 27g/L

Add the component to distilled water and autoclaving for 15 min at 121ºC.

iv. Chromo azurol S agar medium (CAS)

CAS agar was prepared from four solutions which were sterilized before mixing.

Solution 1 – Fe-CAS solution

- 1mM $FeCl_3.6H_2O$ (in 20 mM HCl): 10 ml

- CAS: 60.5 mg in 50 ml of deionized water
- CTAB: 72,9 mg in 40 ml of deionized water

This solution was prepared by mixing 10 ml of the iron solution (1mM $FeCl_3.6H_2O$) with 50 ml of the aqueous solution of CAS. The dark purple mixture resulting was added slowly, with contant stirring, to 40 ml of the aqueous solution of CTAB. This yielded a dark blue solution which was autoclaved for 15 min at 121ºC, then cooled to 50ºC.

Solution 2 – Buffer solution

- PIPES: 30.24 g
- KH_2PO_4: 0.3 g
- NaCl: 0.5 g
- NH_4Cl: 1.0 g
- Agar: 15 g

The PIPES was dissolved in 750 ml of the solution containing the KH_2PO_4, NaCl and NH_4Cl. The pH was adjusted to 6.8 with 50% KOH, and water was added to bring the volume to 800 ml. The solution was autoclaved after adding agar, then colled to 50ºC.

Solution 3 – Micronutrients

- Glucose: 2 g
- Mannitol: 2 g
- $MgSO_4.7H_2O$: 493 mg
- $CaCl_2$: 11 mg
- $MnSO_4.H_2O$: 1.17 mg
- H_3BO_3: 1.4 mg
- $CuSO_4.5H_2O$: 0.04 mg
- $ZnSO_4.7H_2O$: 1.2 mg
- $NaMoO_4.2H_2O$: 1.0 mg

All the components were dissolved in 70 ml of distilled water and autoclaved, then cooled to 50ºC.

Solutions 4 – Casamino acids

- Casamino acids (w:v) 10%

The 30 ml of the solution was filter-sterilized.

After all the solution cooled to 50ºC the solution 3 was added to the buffer solution (solution 2) along with the solution 4. The solution 1 was added last, with sufficient stirring to mix the ingredients without forming bubbles.

v. Pikovskaya medium

- Glucose: 10 g/L
- $Ca_3(PO_4)_2$: 5 g/L
- NaCl: 0,2 g/L
- $(NH_4)_2SO_4$: 0,5 g/L
- Extracto de levedura: 0,5 g/L
- $MnSO_4$: 0,1 g/L
- Agar – Agar: 20 g/L
- $MgSO_4$: 0,1 g/L

Add all the components to distilled water, except $Ca_3(PO_4)_2$ (calcium phosphate)*. Then, autoclaved for 15 min at 121ºC. After cooling, the $Ca_3(PO_4)_2$ was added to the solution.

*The $Ca_3(PO_4)_2$ was sterilized in stove.